



Published in final edited form as:

*Clin Chem.* 2016 January ; 62(1): 287–292. doi:10.1373/clinchem.2015.247148.

## MALDI-TOF-MS Assay to Detect the Hemizygous 22q11.2 Deletion in DNA from Dried Blood Spots

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### Abstract

**Background**—A hemizygous deletion of 1.5–3 Mb in 22q11.2 causes a distinct clinical syndrome with variable congenital defects. Current diagnostic methods use fluorescent in situ hybridization (FISH) or comparative genomic hybridization by microarray to detect the deletion. Neither method is suitable for newborn screening (NBS), since they cannot be performed on dried blood spots (DBS). We developed a MALDI-TOF-MS assay that uses DBS to measure the hemizygous deletion of *UFDIL*, located within the 22q11.2 region.

**Methods**—We used DBS from 54 affected patients, previously tested by FISH or microarray, and 100 cord blood samples to evaluate the performance of the MALDI-TOF-MS assay. With a single primer pair, a 97-base oligonucleotide within *UFDIL* was amplified, as was a sequence on chromosome 18 that differs by 2 nucleotides. A multiplexed, single-base extension reaction created allele-specific products for MALDI-TOF-MS detection. The products were spotted onto a silicon chip, and the height of the spectral peaks identified the relative amounts of target and reference gene.

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**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article

**Authors' Disclosures or Potential Conflicts of Interest:** Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

**Employment or Leadership:** R.F. Vogt, Centers for Disease Control and Prevention.

**Consultant or Advisory Role:** L.J. Kobrynski, APHL and Immune Deficiency Foundation; R.F. Vogt, CLSI.

**Stock Ownership:** None declared.

**Honoraria:** None declared.

**Expert Testimony:** None declared.

**Patents:** None declared.

**Results**—The median ratio of the spectral peak for each *UFDIL* target: reference base was 0.96 and 0.99 for controls, compared with 0.35 and 0.53 for 22q11 deletion syndrome patients. There was 100% concordance between FISH/micro array and MALDI-TOF-MS in all patients with 22q11.2 deletion syndrome.

**Conclusions**—This method can be reliably performed with DBS and is suitable for high sample throughput. This assay may be considered for use in population-based NBS for 22q11.2 deletion.

Newborn screening (NBS)<sup>4</sup> for severe combined immunodeficiency (SCID), with quantitative PCR to detect severe T cell lymphopenia, has been implemented in >30 states (1). Many infants with pathologic T cell receptor excision circles on NBS and T cell lymphopenia on flow cytometry will have a hemizygous deletion in chromosome 22q11 (2–5). The 22q11.2 deletion syndrome (22q11DS), also called velocardial facial syndrome and DiGeorge syndrome, is the most common microdeletion syndrome in humans, with an estimated prevalence of 1 in 3000–4000 live births (6, 7). Many infants are diagnosed prenatally or at birth by the presence of a cardiac defect; others may not be diagnosed until the second decade of life because of the highly variable clinical phenotype. Some degree of T cell lymphopenia due to hypoplasia or aplasia of the thymus gland affects 80% of patients (8–10).

In velocardial facial syndrome/DiGeorge syndrome, most individuals (approximately 90%) have a 3-million-base-pair (3 Mb, or 3 megabase) de novo deletion on 1 allele at chromosome 22q11, and another 7% have a smaller deletion of 1.5 Mb, which overlaps the proximal end of the larger deleted region (11). Current diagnostic tools include fluorescent in situ hybridization (FISH), by use of a probe for *HIRA* (histone cell cycle regulator, formerly *TUPLE1*)<sup>5</sup> located within the common deleted region, or a genome-wide comparative genomic hybridization assay that detects microdeletions of >50 kb (12). Genomic single nucleotide polymorphism arrays can also identify copy number variations in the 22q11.2 gene (13, 14). These assays require whole blood and are labor intensive and expensive to perform. In contrast, quantitative real-time PCR to detect low or absent T cell receptor excision circle copy numbers can be performed on newborn dried blood spots (DBS) (4, 15). Among 3 030 083 infants screened, 29% of infants with pathologic SCID NBS were diagnosed with a non-SCID primary lymphopenia caused by 22q11DS (16). The majority of patients with 22q11DS will have mild T cell lymphopenia at birth and will not be identified through screening for SCID.

Early diagnosis of all affected infants through NBS would permit identification and intervention before potentially life-threatening complications can occur and would improve access to care and services for these children. Professional groups and families of children with 22q11DS have advocated adding NBS for this deletion to the recommended panel (17).

<sup>4</sup>Nonstandard abbreviations: NBS, newborn screening; SCID, severe combined immunodeficiency; 22q11DS, 22q11.2 deletion syndrome; Mb, megabase; FISH, fluorescent in situ hybridization; DBS, dried blood spots; CB-DBS, cord blood DBS; SND, single nucleotide difference; MS, mass spectrometry

<sup>5</sup>Human genes: *HIRA*, histone cell cycle regulator; *UFDIL*, ubiquitin fusion degradation 1 like (yeast); *ZNF74*, zinc finger protein 74; *CFTR*, cystic fibrosis transmembrane conductance regulator (ATP-binding cassette subfamily C, member 7)

Other PCR methods that use DNA extracted from whole blood or cell lines, including real-time quantitative PCR, quantitative interspecies competitive PCR, competitive fluorescent multiplex short tandem repeat polymorphism assay, and multiplex ligation-dependent probe amplification, have been shown to identify the hemizygous deletion of 22q11.2 compared with a reference gene (18–25). These approaches require complex algorithms to compensate for variable amplification efficiency of the reference and target sequences, which could introduce imprecision and affect interpretation of results. Here we describe the development of a novel assay for newborn screening of 22q11.2DS on DBS samples that combines PCR and MALDI-TOF-MS to identify hemizygous deletion of a gene located within the 22q11.2 common deleted region.

## Participants and Methods

### Participants

We collected whole blood in EDTA tubes from 54 patients with 22q11DS diagnosed previously by FISH or comparative genomic hybridization microarray. All patients gave informed consent to the institutional review board–approved protocol at Emory University. To prepare DBS, 100  $\mu$ L whole blood was spotted on Guthrie cards, air-dried for at least 3 h, and kept at  $-20^{\circ}\text{C}$  in a sealed plastic bag with desiccant packs and a humidity card. We prepared control DBS samples from anonymous cord blood samples (CB-DBS) and unaffected parents of patients in the 22q11DS clinic.

### Selection Of Pcr Targets

For the 22q11.2 target, we chose *UFDIL* [ubiquitin fusion degradation 1 like (yeast)], which is located in the common deleted region of chromosome 22. Potential internal reference candidate genes were identified by searching human DNA with GenBlast (26) for regions containing sequences of 90–150 nucleotides with extensive homology to *UFDIL*. A region with 90% homology was located in an intergenic region on chromosome 18 (27); this 97-nucleotide sequence differs by only 2 bases from the target *UFDIL* sequence (Table 1). No other homologous regions were identified within the human genome database. Forward and reverse primers (Table 1), which could amplify both the target and reference sequences, were designed with Primer Express v2.0 (Applied Biosystems). Primer specificity was checked on the University of California Santa Cruz Genome Bioinformatics in silico PCR (28). We added a 10mer segment (AGGATGAAGC) at the 5' end of the primer sequences to increase the mass of the primers and prevent interference with spectrometric measurement of the final products.

### Quantification By Maldi-Tof-Ms

The 97-bp product from the first PCR reaction served as the template for the extension PCR. We multiplexed 2 separate 17-base extension primers, each ending just before a single nucleotide difference (SND) that distinguished the target *UFDIL* (chr22) amplicon from the internal reference (chr18) amplicon (Table 1). The first SND was a C>T change (SND\*1); the second was a C>A change (SND\*2). The 18-base extension products were distinguishable by their unique masses measurable by mass spectrometry (MS) (Table 1). The use of mass-modified dideoxy nucleotide terminators (Agena Bioscience), included in

the reaction mixture, truncated the extension reaction after the addition of 1 base. The relative quantity of the products was represented by the height of the spectral peaks at the expected mass.

We used 2 extension primers in a multiplex reaction to generate 2 independent sets of extension products (Table 1). This was intended to provide corroborating results.

## PCR

After preliminary studies for optimization, the following protocol was found to produce reliable and reproducible results. Genomic target and reference sequences were amplified directly from the DBS punches. DBS 2.0-mm discs were punched into individual PCR tubes in a 96-well plate. Each sample was washed once with 100  $\mu$ L DNA purification solution (Qiagen) with shaking (1500 rpm) for 10 min at room temperature. After removing the wash buffer, the disc was washed with 100  $\mu$ L DNA elution buffer for 10 min. The buffer was discarded, and a PCR reaction mix containing PCR buffer (Agena Bio-science),  $MgCl_2$  (2 mmol/L), dNTP mix (500  $\mu$ mol/L), hot start *Taq* polymerase (0.5 U) (Agena), and forward and reverse primers (800  $\mu$ mol/L) in a final volume of 15  $\mu$ L was added to each tube. PCR amplification was performed in a Bio-Rad C1000 thermocycler, starting with DNA denaturation at 94 °C for 10 min, followed by 45 cycles of amplification consisting of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. The final incubation was at 72 °C for 5 min. After PCR, the reaction products were stored at 4 °C or used immediately for the reaction to generate extension products for MALDI-TOF-MS analysis.

## Single Base Extension

To generate the extension products, we transferred 5  $\mu$ L of each sample (primary PCR product) to an individual well in a 384-well plate. Unincorporated dNTP and primers from the PCR reaction were removed by incubating with 0.5 U shrimp alkaline phosphatase at 37 °C for 40 min, then at 85 °C for 5 min. We added 2  $\mu$ L extension reaction mix, containing PCR buffer, polymerase enzyme, mass-modified ddNTP terminators (iPLEX reagents, Agena Bioscience), and extension primers (1.04  $\mu$ mol/L), to each well. The plate was sealed, placed in a thermocycler, and heated at 94 °C for 30 s to denature the DNA in the sample. The thermocycler program used 2 loops: heating to 94 °C for 5 s to denature the DNA, then a set of 5 cycles of 52 °C for 5 s and 80 °C for 5 s for annealing and extension. These 2 loops were repeated for 40 cycles so that 200 short cycles were completed. The final extension step was 72 °C for 3 min.

The reaction products were desalted by mixing with 16  $\mu$ L water and 6 mg ion-exchange resin (Agena Bioscience) for 30 min. The plates were centrifuged for 5 min at 2000g to pellet the resin to the bottom of the tubes. We used a robotic benchtop nanodispenser (Agena Bioscience) to transfer 20 nL supernatant sample to individual pads on a 384-element silicon chip matrix (SpectroCHIP®).

## Maldi-Tof Analysis

The samples on the SpectroChip were analyzed on the MSArray workstation (Agena Bioscience) (29) with a MALDI-TOF mass spectrometer (MassArray analyzer compact,

Agena Bioscience) containing a Class I nitrogen laser emitting 40–90 microjoules at 337 nm. A calibration spectrum was acquired for each chip with the 3-point calibrators provided with the system. Spectral measurements were acquired for the center and 4 other positions around each matrix pad. We used MassArray Typer4.0 (Agena Bioscience) software to analyze the data from the spectral peaks to determine the genotyping call for each sample.

## Results

MALDI-TOF-MS analysis generated results in a spectrogram providing signal peaks arranged by oligonucleotide mass, with signal intensity data. The assay was able to discriminate between spectral peaks representing sequences that differed by 14 Da. All of the tested samples from 22q11.2DS patients and unaffected controls exhibited 4 distinct peaks, located at the expected mass of the 4 extension products (Fig. 1). The masses of the extension oligonucleotides from the target sequences (*UFDIL* on chromosome 22) were 5513 Da for SND\*1 and 5530 Da for SND\*2 (Table 1). Those from the internal-reference sequences (on chromosome 18) were 5593 Da for SND\*1 and 5554 Da for SND\*2. For each sample, the ratio of the peak intensities for each SND from the target 22q11.2 sequence on chromosome 22 to that of the reference sequence on chromosome 18 (representing 2 copies per cell) was used. A ratio close to 1.0 was expected from healthy (2N) controls, whereas a ratio close to 0.5 would indicate a hemizygous deletion (1N).

Initial experiments with 2 separate cohorts, 10 CB-DBS and 10 22q11DS DBS, demonstrated good internal consistency. The median (SD) ratio of peak signal intensity for SND\*1 was 1.06 (0.07) for the first cohort and 1.15 (0.12) for the second cohort of cord blood controls; the ratios of target: reference sequence peak signal intensity were significantly different ( $P < 0.001$ ) at 0.55 (0.01) and 0.6 (0.04) for 2 groups of affected patients. Similarly, the SND\*2 median ratios (SD) of peak intensity were 0.98 (0.06) and 0.89 (0.03) for controls and 0.56 (0.06) and 0.51 (0.03) for 22q11DS samples (Fig. 2). There was no overlap between the signal ratio for any sample from the control and 22q11DS groups. Subsequent experiments demonstrated some variation in the peak intensity between runs, with the median peak ratio 1.06–1.45 for controls and 0.56 – 0.88 for 22q11DS samples. However, the signal ratio for controls was significantly different from 22q11DS samples, without overlap ( $P < 0.001$ ) within any run.

To evaluate the validity of the assay in a larger sample size, we tested 100 additional CB-DBS samples and 54 DBS from 22q11DS patients. Fig. 3 shows the ratios of signal intensity between *UFDIL* and the reference gene on chr18 for each SND, reflecting the relative gene copy number. The ratio values were normalized to 1.0 with a set of 4 standard samples that were run in each experiment to compensate for signal drift between runs. In the CB-DBS (with 2 copies of target sequence), the median ratio was 0.96 for SND\*1 and 0.99 for SND\*2. For the 22q11DS patients (1 copy of target sequence), it was 0.36 for SND\*1 and 0.53 for SND\*2. A cutoff value of 0.7 showed 100% diagnostic sensitivity and specificity, correctly identifying all patients with the deletion. None of the control samples were outside the reference range.

## Discussion

Multiplex competitive PCR with MALDI-TOF can measure copy number variation at multiple loci over a wide mass range, with as little as 20 ng DNA (30). Our report describes the application of MALDI-TOF-MS to detect the hemizygous deletion in 22q11.2 by use of DNA extracted from DBS. The established MALDI-TOF-MS approach for analysis of gene copy number (iPLEX assay, Sequenom) is based on competitive PCR with an external synthetic oligonucleotide competitor that is nearly identical to the target (30). It requires multiple concentrations of the competitor for each sample (thus multiple tests), since the optimal concentration is usually unknown and may vary from sample to sample. The assay result in copy concentration must be converted to copies per cell, usually through logarithmic transformation or linear regression on the basis of the DNA concentration of the competitor. The critical modification in our assay is the use of an internal genomic sequence as the competitor, which assures that the ratio of competitor to target is naturally within optimal range (2:1 for hemizygous deletion, 1N; 2:2 for no deletion, 2N), regardless of the variable amount of host DNA that may be in the sample DBS. The result does not need complicated conversion because it is normalized to the internal genomic reference sequence, which is always present in 2 copies per cell.

In theory, the assay could be further expanded by use of additional targets in a multiplex format to distinguish the smaller 1.5-Mb deletions from the more common 3.5-Mb deletion. One possible candidate is *ZNF74* (zinc finger protein 74) located outside the 1.5-Mb deleted region. In a preliminary experiment, a sample obtained from a patient with the 1.5-Mb deletion was found to produce a ratio of peak intensity of 0.55 for *UFD1L* but 1.0 for *ZNF74*. This result agreed with the diagnostic chromosomal microarray assay showing that the deletion was 1.5 Mb in size and that *ZNF74* was not affected in this patient.

Other advantages are associated with this design of MALDI-TOF-MS assay that uses an internal competitor. It requires a small amount of DNA (a 2-mm DBS punch yields approximately 100 ng DNA (31)); it requires a single set of PCR and extension primers for both target and reference genes, ensuring equal amplification efficiency; and detection of the product does not require the use of an oligonucleotide fluorescent reporter probe. Up to 384 samples could be run on a single chip, allowing high throughput. Robotic automated operation with liquid handlers and droplet dispensers at the pre- and post-PCR processes could greatly reduce the manpower needed to perform this assay.

The excellent resolution of MS allows a very high level of assay multiplexing, which can occur at the PCR stage or after PCR by combining products from multiple PCR reaction tubes for MALDI-TOF-MS analysis. Use of MALDI-TOF-MS analysis of *CFTR* [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette subfamily C, member 7)] for 180 mutations simultaneously in NBS has recently been reported (32).

In summary, we describe the use of MALDI-TOF-MS for detecting a hemizygous deletion by use of an internal genomic sequence as competitor. Testing DNA extracted from DBS, we found that the assay discriminated all 54 22q11DS samples from control samples. Although these results are highly promising, full clinical validation of this MALDI-TOF-MS assay



will require testing a large population-based sample of newborn DBS before it can be applied to routine newborn screening.

## Acknowledgments

**Research Funding:** L.J. Kobrynski, National Institute of Allergy and Infectious Diseases and NIH (R03A1-07976502).

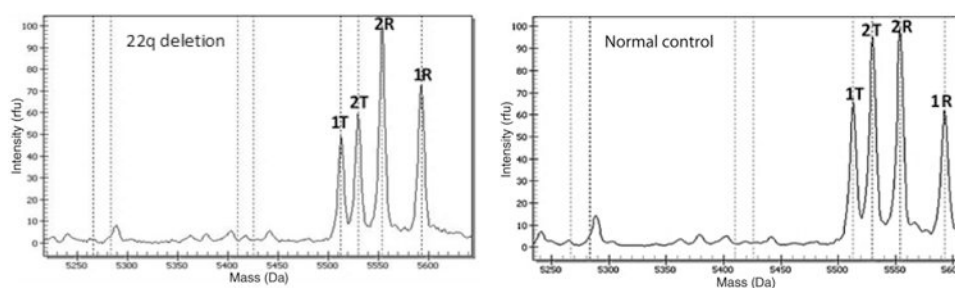
**Role of Sponsor:** The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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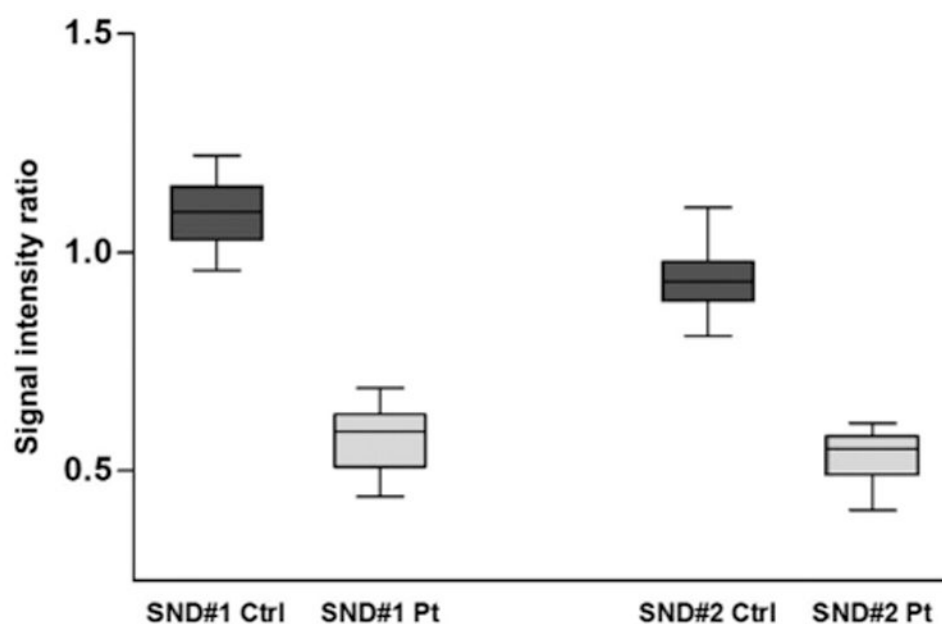
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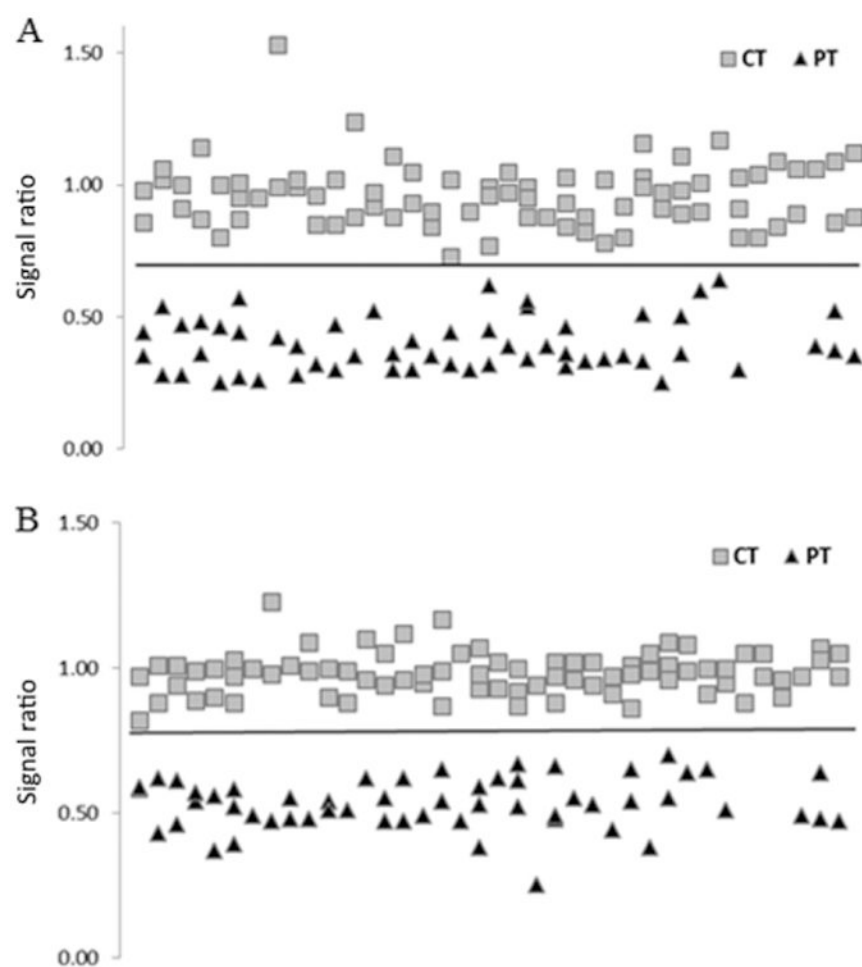


**Fig. 1. Mass spectrograms for 22q11DS sample and sample without 22q11 deletion (healthy control)**

T, target gene; R, reference gene.



**Fig. 2.** Box and whisker (Tukey) plot of median signal ratio at SND #1 and SND #2 for a cohort of 10 22q11DS samples and 10 nondeleted samples  
Ctrl, control; Pt, patient.



**Fig. 3.** Signal ratios for samples from 100 unaffected individuals (CT) and 54 patients with 22q11.2 DS (PT) at (A) SND\*1 and (B) SND\*2.

Table 1

Sequences of the 97-base PCR amplification product and extension products for SND regions 1 and 2.<sup>a</sup>

| Primer or product           | Sequence, 5' to 3'   | Mass, Da |
|-----------------------------|--|----------|
| Forward primer <sup>a</sup> | ACGTTGGATGGGATGAAGCTGGAGGCAGATT  |          |
| Reverse primer <sup>a</sup> | GATTCACTCTTGACAACCGACTAAGTAGGTGGCA   |          |
| Product                     | AGGATGAAGCTGGAGGCAGATTCTGTCAT TG[C/A]GTAAAAAGGGAAGAAAGCCCTAAGTGAGGACTGTTGGCTGATT |          |
| SND*1 <sup>b</sup>          |  |          |
| Chr22                       | GCTGGAGGCAGATTCGTC   | 5513     |
| Chr18                       | GCTGGAGGCAGATTCGTT   | 5593     |
| SND*2 <sup>b</sup>          |  |          |
| Chr22                       | GAGAAAGGACAGTCATTGC  | 5530     |
| Chr18                       | GAGAAAGGACAGTCATTGA  | 5554     |

<sup>a</sup>The 10mer oligonucleotide added to the primers is underlined; the SND of the target and reference sequences is in brackets.

<sup>b</sup>The difference in the terminal base is underlined.